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Single-Assay Combination of Epstein-Barr Virus (EBV) EBNA1- and Viral Capsid Antigen-p18-Derived Synthetic Peptides for Measuring Anti-EBV Immunoglobulin G (IgG) and IgA Antibody Levels in Sera from Nasopharyngeal Carcinoma Patients: Options for Field Screening

J. Fachiroh,[1] D. K. Paramita,[1] B. Hariwiyanto,[1] A. Harijadi,[1] H. L. Dahlia,[1] S. R. Indrasari,[1] H. Kusumo,[1] Y. S. Zeng,[2] T. Schouten,[3] S. Mubarika,[1] and J. M. Middeldorp[3]*

Faculty of Medicine, Gadjah Mada University and Sardjito Hospital, Yogyakarta, Indonesia[1]; Department of Virology, Chinese Academy for Preventive Medicine, Beijing, China[2]; and Department of Pathology, Free University Medical Center, Amsterdam, The Netherlands[3]

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Assessment of immunoglobulin A (IgA) antibody responses to various Epstein-Barr virus (EBV) antigen complexes, usually involving multiple serological assays, is important for the early diagnosis of nasopharyngeal carcinoma (NPC). Through combination of two synthetic peptides representing immunodominant epitopes of EBNA1 and viral capsid antigen (VCA)-p18 we developed a one-step sandwich enzyme-linked immunosorbent assay (ELISA) for the specific detection of EBV reactive IgG and IgA antibodies in NPC patients (EBV IgG/IgA ELISA). Sera were obtained from healthy donors ($n = 367$), non-NPC head and neck cancer patients ($n = 43$), and biopsy-proven NPC patients ($n = 296$) of Indonesian and Chinese origin. Higher values of optical density at 450 nm for EBV IgG were observed in NPC patients compared to the healthy EBV carriers, but the large overlap limits its use for NPC diagnosis. Using either EBNA1 or VCA-p18 peptides alone IgA ELISA correctly identified 88.5% and 79.8% of Indonesian NPC patients, with specificities of 80.1% and 70.9%, whereas combined single-well coating with both peptides yielded sensitivity and specificity values of 90.1 and 85.4%, respectively. The positive and negative predictive values (PPV and NPV, respectively) for the combined EBNA1 plus VCA EBV IgA ELISA were 78.7% and 93.9%, respectively. In the Indonesia panel, the level of EBV IgA reactivity was not associated with NPC tumor size, lymph node involvement, and metastasis stage, sex, and age group. In the China panel the sensitivity/specificity values were 86.2/92.0% (EBNA1 IgA) and 84.1/90.3% (VCA-p18 IgA) for single-peptide assays and 95.1/90.6% for the combined VCA plus EBNA1 IgA ELISA, with a PPV and an NPV for the combined EBV IgA ELISA of 95.6 and 89.3%, respectively. Virtually all NPC patients had abnormal anti-EBV IgG diversity patterns as determined by immunoblot analysis. On the other hand, healthy EBV carriers with positive EBV IgA ELISA result showed normal IgG diversity patterns. By using EBV IgG immunoblot diversity as confirmation assay for EBV IgA ELISA-positive samples, the sensitivity and specificity for NPC diagnosis increased to 98% and 99.2%, respectively, in the Indonesian NPC samples. The use of these combined methods for seroepidemiological screening studies is proposed.

Nasopharyngeal carcinoma (NPC) is a highly prevalent malignancy in southern China, most of Southeast Asia, and north Africa (36, 48). In Indonesia, especially in central Java, undifferentiated NPC (WHO type III) also ranks among the most common cancers. For instance NPC is ranked 1 in males and 3 in females in the Yogyakarta province (38), with regional villages representing hot spots of NPC incidence.

The consistent expression of Epstein-Barr virus (EBV) gene products in NPC tumor cells (33, 36, 47, 48) and the distinct serological responses to defined EBV antigens in NPC patients illustrate the close association between EBV and this disease (18, 19, 37, 49). The mucosal origin of NPC is reflected by characteristic immunoglobulin A (IgA) responses in NPC patients (13). Of the latency-associated EBV gene products expressed in NPC tumor cells only EBNA1 induces strong IgG and IgA antibody responses; LMP1, LMP2, and BARF1 do not (28, 42). On the other hand NPC patients have strong and characteristic antibody responses against EBV lytic-cycle proteins, including early (EA) and viral capsid (VCA) antigen complexes. Lytic-cycle products are rarely expressed in the NPC tumor cells but may originate from viral replication in differentiating NPC cells (51). The assessment of anti-EBNA1, anti-EA, and anti-VCA antibodies requires separate assays, each contributing to NPC diagnosis (23). Individual NPC patients may respond quite differently to EBV lytic-cycle proteins, but the small capsid protein VCA-p18, encoded by the BFRF3 reading frame, is a highly prevalent target for antibody responses, including IgG and IgA (3, 9, 13, 23, 35, 44). However, high titers of IgG against EA/VCA are not specific for NPC and can be observed in other EBV-linked diseases as well (18). Elevated IgA antibodies against EA/VCA and nuclear antigens, especially to EBNA1 (collectively referred to as EBV IgA), present an outstanding feature of NPC patients (10, 16). The molecular diversity of EBV antigens recognized by IgG and IgA antibodies differs between individuals and increases with tumor stage. Moreover the diversity of antigen recognition between IgG and IgA responses

* Corresponding author. Mailing address: Dept. Pathology, Vrije Universiteit Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. Phone: 31-20-4444052. Fax: 31-20-4442964. E-mail: j.middeldorp@vumc.nl.

within an individual NPC patient may also differ significantly, suggesting independent triggering of IgG- and IgA-producing B cells (13). Importantly, the presence of EBV IgA antibodies is associated with increased NPC risk in the general population (6, 10, 50). Therefore it was suggested that field screening for EBV IgA might be useful to identify patients with early-stage NPC (50). In addition, in established NPC patients, longitudinal monitoring of EBV IgA reactivity levels may be used for prognosis, because declining reactivity is associated with remission and stable or increasing responses are associated with persistent or recurrent disease and development of metastasis (34).

Currently indirect immunofluorescence assay (IFA) methods are still widely used as the "gold standard" for EBV serodiagnosis in NPC (24, 36). IFA, however, is time-consuming, not well standardized, and ill suited for large-scale testing or automated handling. Enzyme-linked immunosorbent assay (ELISA) techniques provide a promising alternative with potential for automation and mass screening However, ELISA methods described to date have used a variety of EBV antigens, and standardization has not been achieved yet (17). Standardized EBV IgA ELISAs suited for field screening require the availability of high-quality, reproducible, and preferably cheap EBV antigens comprising the immunodominant markers for EBV IgA antibodies. Previously reported EBV antigens for ELISA include EBV cell extracts (11, 12, 46), purified native or recombinant EBV proteins (3, 7, 9), and synthetic peptides (16, 23, 44). Among the defined EBV antigens proposed as markers in ELISA are thymidine kinase (7, 25), DNase (41), ribonucleotide reductase (15), ZEBRA (9, 22), VCA-p18 (35, 44), EBNA1 (16, 21), and EAd-p47/p138 (14). However, single markers may not be sufficient to identify all individual NPC patients in view of the observed diversity of antibody reactivity among individual NPC patients. Combined testing for ZEBRA and VCA-p18 IgA or ZEBRA plus EBNA1 IgA reactivity was recently proposed for more-sensitive NPC diagnosis (1, 2, 9). In all cases two-marker testing required testing by two separate assays.

A single assay combination for simultaneous analysis of IgA/IgG antibody responses to multiple EBV proteins is provided by immunoblot testing using either extracts from EBV producer cells (13) or combinations of recombinant proteins as used in commercial line blot assay (17). More recently a combination of EBV IgA serology and EBV DNA quantification was proposed (2, 24, 26). However, these assays are laborious and expensive and therefore not well suited for mass screening.

In this study we describe the development of an IgA ELISA for the primary diagnosis of NPC by combining EBNA1- and VCA-p18-derived multiepitope synthetic peptides in a single-well format. Synthetic peptides have the advantage of being chemically defined, allowing reproducible large-scale production at low costs and improved assay standardization (45). The combination of two immunodominant antigens for EBV IgA detection holds promise for development of a simple NPC screening and monitoring assay. EBV immunoblot IgG diversity analysis, recently shown by us to provide detailed molecular and diagnostically relevant information for NPC serology, was used as a confirmatory assay (13).

MATERIALS AND METHODS

Sera. The Indonesia serum panel consisted of sera from 147 histologically confirmed NPC patients, 43 non-NPC patient controls (22 with other head and neck cancer, 21 with breast cancer), all collected at Sardjito General Hospital, and from 254 healthy donors, obtained from the local Red Cross blood bank (n = 180) and hospital/university staffs (n = 74) at Gadjah Mada University, Yogyakarta, Indonesia. The NPC sera were taken on the first visit of patients to the ear, nose, and throat department (ENT) during the years 2001 to 2003. NPC staging was done by ENT examination and computed tomography scan and classified according to the 1996 Union International Cancer Control classification. From all the NPC patients that enrolled at ENT, postnasal and/or lymph node biopsy samples were obtained and confirmed for the presence of undifferentiated carcinoma cells and the presence of EBV by EBV-encoded small RNA in situ hybridization using a peptide nucleic acid probe (Dakopatts, Glostrup, Denmark) or by immunohistochemistry (Labvision Corp., Fremont, CA) using EBNA1- and LMP1-specific monoclonal antibodies OT1X (5) and OT21C (28, 29) or both tests.

The China serum panel consisted of sera from 183 confirmed NPC patients and 113 regional healthy controls from south China (10).

A panel of sera from infectious mononucleosis (IM; n = 39) and chronic EBV (C-EBV) patients (n = 10) and healthy donors (n = 6) from The Netherlands were used as controls from a nonendemic area. These reference sera were obtained from the archives of the VU University Medical Center in Amsterdam, The Netherlands (28). All sera were stored at −20°C until use.

EBV synthetic peptides. Immunodominant epitopes on VCA-p18 and EBNA1 were defined as described before (31, 32, 44). Briefly, from the predicted amino acid sequence encoded by the BFRF3 (VCA-p18) and BKRF1 (EBNA1) open reading frames on the EBV genome, all possible 12-mer peptides with an overlap of 11 were synthesized on polypropylene pins and tested by PEPSCAN analysis as described by Middeldorp and Meloen (31). Immunodominant epitopes for VCA-p18 (BFRF3) were located at amino acids 119 to 148 and 153 to 176 (44), and for EBNA1 (BKRF1) such epitopes were located at 382 to 410 and 413 to 452 (32). Individual 30-mer polyepitope peptides spanning the respective sequence were synthesized by 9-fluorenylmethoxy carbonyl chemistry, purified by high-performance liquid chromatography (HPLC), and subsequently linked by S-S bond via two additional terminal cysteines at the C and N termini coupled during synthesis (Neosystem, Strassbourg, France). The S-S-linked peptides were once more purified by HPLC to achieve >90% purity, dried, and stored at −20°C until use.

ELISA. Ninety-six-well ELISA plates (Greiner Labortechniek, Germany) were coated with EBNA1 and VCA-p18 peptides using 135 μl single peptide (1 μg/ml) or combined peptides (1 μg/ml EBNA1 plus 0.5 μg/ml VCA-p18) in 0.05 M Na_2CO_3, pH 9.6 (Merck, Darmstadt, Germany). After overnight incubation at 4°C, antigen was discarded and 200 μl blocking buffer (3% bovine serum albumin [BSA; Roche Diagnostic GmBH, Germany] in phosphate-buffered saline [PBS]) was added to each well. After a 1-h incubation at 37°C, the wells were emptied and washed three times with PBS containing 0.05% Tween 20 (PBS-T). Subsequently, 100 μl 1:100-diluted serum was applied (serum dilutions in PBS-T, 1% BSA, 0.1% Triton X-100) and incubated for 1 h at 37°C. All sera were tested in duplicate. After four washings with PBS-T, rabbit anti-human IgA-horseradish peroxidase (HRP) conjugate (diluted 1:4,000 in serum dilution buffer) or anti human IgG-HRP conjugate (diluted 1:6,000 in serum dilution buffer) (Dako) was added and incubated for 1 h at 37°C. After four washes with PBS-T, 100 μl/well of 5′5′,3′3′tetramethylbenzidine substrate solution (bioMerieux, Boxtel, The Netherlands) was added and kept in the dark for 30 min (for IgA detection) or 10 min (for IgG detection). The reaction was stopped by adding 100 μl of 1 M H_2SO_4 (Merck, Schuchardt, Germany). The optical density was determined at 450 nm (OD_{450}) using an ELISA reader (2001; Anthos, Austria). All OD_{450} values were normalized by subtracting the value for 1:100-diluted EBV-negative sera used in duplicate in each ELISA run. The receiver operating characteristic (ROC) curve was drawn to determine the cutoff values (CoV) for EBNA1, VCA-p18, and EBNA1 plus VCA-p18 (EBNA1+VCA-p18) by using a large panel of Indonesian healthy subjects (n = 254) and NPC patients (n = 151). CoV were applied to determine diagnostic specificity and sensitivity (7). In initial studies prior to IgA ELISA analysis sera were pretreated with GullSorb (Meridian, The Netherlands) as described by the manufacturer in order to remove possibly competing IgG antibodies.

Immunoblot detection. Immunoblot strips containing HH514.c16 nuclear antigen induced chemically to produce the late lytic phase of EBV proteins were used to detect IgG antibody to EBV proteins. The strips were prepared and analyzed exactly as described by Middeldorp and Herbrink (30). To determine the position of characteristic EBV antigens, blot strips were incubated with

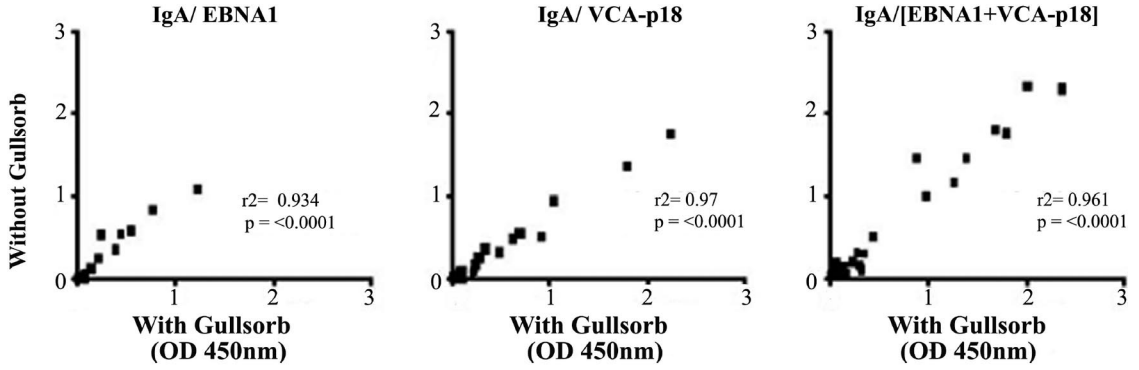

FIG. 1. The effect of GullSorb in EBV peptide IgA ELISA. Twenty-two distinct sera from confirmed NPC cases having variable IgG and IgA reactivities to EBV antigens, as defined in a previous study (13), were tested in three separate EBV peptide ELISAs for IgA reactivity either with or without prior GullSorb treatment using the protocol prescribed by the manufacturer. Correlation coefficients (R^2) >0.9 for IgA ELISA results show that prior removal of IgG is not required, indicating that EBV-specific IgG and IgA antibodies in NPC sera are not competing and appear to recognize different epitopes. All assays were done in parallel, and identical results were obtained in repeated experiments.

monoclonal or polyclonal antibodies of known specificity and human reference sera and the EBV-specific antibody diversity profiles of IgG and IgA responses were used as confirmation of the IgA ELISA as recently described (13, 23).

Statistical analysis. All statistical analysis was done by the GraphPad Prism, ver. 4.0, program. ROC analysis was done to determine CoV; one-way analysis of variance was used to compare mean values of different antigens in IgA ELISA and differences in IgA ELISA values in relation to tumor size, lymph node involvement, and metastasis (TNM) stage, age, and sex group; and linear regression analysis was used to determine the use of Gullsorb.

RESULTS

EBV peptide ELISA. In a first series of experiment conditions for peptide antigen coating, serum and conjugate dilution were optimized (data not shown), leading to the protocol described in Materials and Methods. Optimal detection of IgA antibodies to EBNA1 and VCA-p18 separately required coating at 1 μg/ml. For simultaneous detection of IgA antibodies to EBNA1+VCA-p18 peptides, prior incubation with 1 μg/ml EBNA1 peptide for 2 h at 37°C, followed by subsequent 0.5-μl/ml VCA-p18 peptide overnight proved optimal for preparing the solid phase. Best coating was obtained by using standard 0.05 M $NaHCO_3$ buffer at pH 9.6. Following peptide coating and postcoat blocking with 3% BSA, the coated plates could be dried, sealed, and stored at 4°C without significant loss of reactivity for several months (data not shown). However, in most experiments shown here weekly freshly coated ELISA plates were used.

Effect of IgG removal on IgA antibody reactivity. Because IgG reactivity to EBNA1 or VCA-p18 is present in virtually all sera and might bind identical epitopes as IgA, we compared serum reactivities with and without prior IgG removal using GullSorb, previously shown to be highly effective in removing IgG prior to IgM detection in ELISA (44). Results shown in Fig. 1 reveal that prior IgG removal did not affect the IgA reactivity of NPC sera in EBNA1 or VCA-p18 or combined EBNA1+VCA-p18 ELISA.

Comparison of IgG and IgA ELISA. Using a limited but random selection of serum specimens from each group (healthy subjects, control patients, and NPC patients), we first optimized ELISA conditions for discriminating NPC cases and controls by IgG and IgA peptide-based ELISA. Results showed 100% positive IgG antibody reactivity for EBNA1 and VCA-p18 and for the combination ELISA. The individual OD_{450} values in the combined EBNA1+VCA-p18 IgG ELISA were always higher than for the single-antigen IgG ELISA.

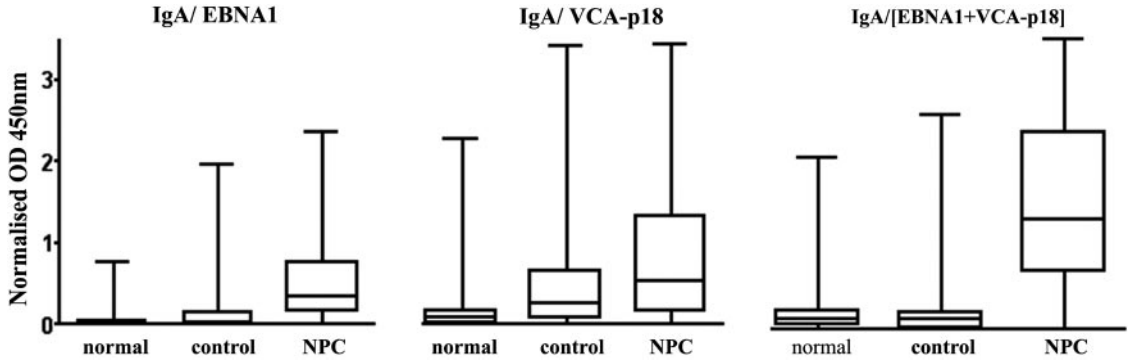

FIG. 2. Distribution frequency of EBV IgA ELISA values in the complete Indonesia panel for EBNA1, VCA-p18, and combination antigen. Shown are the mean OD_{450} values and 25th and 75th percentiles of OD_{450} values for IgA reactivity in three EBV synthetic-peptide ELISAs using Indonesian serum panels consisting of healthy blood donors (n = 254), cancer control cases (n = 43), and NPC patients (n = 151). The results reveal that combined EBNA1+VCA-p18 IgA ELISA provides better discrimination of NPC patients and controls compared to either single-antigen IgA ELISA ($P < 0.0001$).

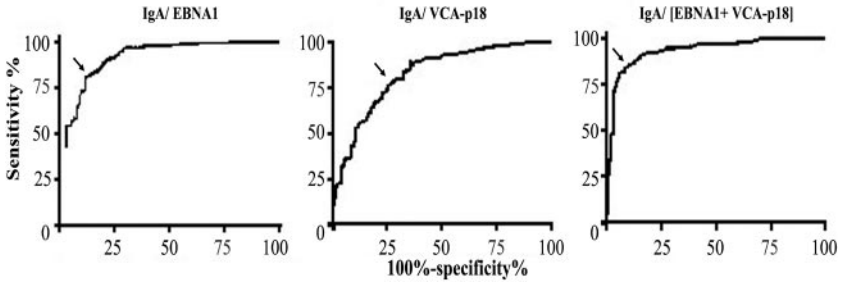

FIG. 3. ROC curve of IgA ELISA using EBNA1 and VCA-p18 peptide as single and combination antigen. The ROC curve plot was made by using the data from a large panel of sera from healthy blood donors ($n = 254$) and NPC patients ($n = 151$) of Indonesian origin. The ROC-determined sensitivity and specificity of the ELISA method yielded PPV and NPV values as detailed in Tables 1 and 2. Arrows show the sensitivity and specificity values chosen for each ELISA system.

The mean IgG reactivities from the EBNA1+VCA-p18 ELISA for healthy carriers, non-NPC patient controls, and NPC patients from Indonesia were reflected in OD_{450} values of 2.43, 1.86, and 3.05, respectively, being significantly higher for NPC patients compared to both control populations ($P < 0.05$). However, the wide overlap in EBV IgG ELISA reactivity between healthy carriers and non-NPC tumor controls versus NPC patients prevented its diagnostic use. For IgA ELISA the difference was more significant ($P < 0.0001$), particularly in the EBNA1 and EBNA1+VCA-p18 combination assays, the latter producing mean OD_{450} values of 0.2, 0.3, and 1.33 for healthy carriers, control patients, and NPC patients, respectively.

EBV IgA ELISA of the large panel. For the further analysis of EBV IgA reactivity we subsequently screened a larger population of healthy carriers from Indonesia ($n = 254$) and NPC patients ($n = 151$) collected over a 3-year period and also included several non-NPC cancer controls ($n = 43$). The mean OD_{450} values for healthy carriers, controls, and NPC patients were 0.05, 0.20, and 0.53, respectively, for EBNA1; 0.17, 0.55, and 0.86, respectively, for VCA-p18; and 0.22, 0.24, and 1.52, respectively, for combination antigens. The mean OD_{450} values for NPC sera were significantly higher in all three IgA ELISAs, with the best discriminatory value for the EBNA1+VCA-p18 combination assay ($P < 0.0001$). Results for individual samples from the larger Indonesian panel (Fig. 2) showed that in some non-NPC tumor control cases VCA-p18 ELISA detection yielded high IgA reactivity, in contrast to the EBNA1 ELISA. However, EBV IgA combination ELISA picked up both signals, leading to reduced overall reactivity but with a significantly improved discrimination between NPC patients and controls ($P < 0.0001$).

In order to develop a reproducible screening assay, we defined the optimal cutoff value by ROC analysis to give the best discrimination between healthy carriers and NPC patients (8). Figure 3 shows ROC analysis of the Indonesian panel of NPC patients ($n = 151$) and healthy regional donors ($n = 254$) used to calculate the optimal CoV for IgA ELISA using either EBNA1 or VCA-p18 or the EBNA1+VCA-p18 combination as the solid-phase antigen. Table 1 shows corresponding CoVs and associated sensitivity and specificity values of IgA ELISAs with single and combination antigens. These results yielded PPV/NPV values exceeding 80% for EBNA1, VCA-p18, and the combination assay, respectively, as shown in Table 2.

Based on the optimum CoV defined by ROC analysis, 37 (14.6%) healthy carriers and 7 (16.3%) control patients showed values above CoV in the combination assay (included two nasal cavity cancer cases, one larynx cancer case, one non-Hodgkin lymphoma case, and three breast cancer cases), while 14 (9.5%) NPC cases showed values below CoV. These individuals were further analyzed by immunoblotting as described below.

NPC patients were further classified according to stage of disease, sex, age, and TNM status and analyzed for their serological response in the EBV IgA ELISA. Most patients in the Indonesia panel presented with advanced disease (87.4%), and there was a 2.63 male/female ratio, with an age distribution of 25 to 80 years (mean, 47.6 years). Our analysis showed no significant correlation ($P > 0.05$) between any of the EBV IgA ELISA values and any of the parameters tested (data not shown). Although the highest EBV IgA responses were observed in the patient with the most advanced stage of disease, the mean and median values were not statistically different from patients with early-stage disease (Fig. 4).

Comparison of IgA ELISAs for Indonesian and Chinese populations. A separate study using identical EBV ELISA conditions was also done in China, using locally collected samples from NPC patients and regional Chinese controls from the Wuzhou City region (10, 50). For this purpose, precoated, dried, and sealed ELISA plates were provided to our Chinese collaborators, together with standard serum diluents and detection reagents in concentrated form plus a work sheet. Overall results for the Indonesia and China panels are shown in Table 2, which revealed that in Chinese individuals the use of single-antigen ELISA gave a similar positive detection rate for both EBNA1 and VCA-p18 IgA as found in the Indonesia panel, whereas the use of the VCA-p18 peptide was less sensitive than EBNA1. Highly comparable results were found

TABLE 1. Cutoff values and diagnostic performance characteristics of EBV IgA ELISA in the Indonesia panel consisting of sera from healthy donors ($n = 254$) and NPC patients ($n = 151$)

Parameter	Value for:		
	EBNA1	VCA-p18	EBNA1+VCA-p18
Cutoff value	0.1205	0.2233	0.3536
Sensitivity (%)	88.6	79.8	85.4
Specificity (%)	80.1	70.9	90.1

TABLE 2. Detection of antibodies by IgA EBNA1 ELISA, VCA-p18 ELISA, and the combination of the two in both Indonesia and China panels

Serum source	EBNA1				VCA-p18			EBNA1+VCA-p18			
	n	No. positive (%)	PPV (%)	NPV (%)	No. positive (%)	PPV (%)	NPV (%)	*n*	No. positive (%)	PPV (%)	NPV (%)
Indonesia panel											
NPC patients	151	121 (80.1)	80.6	88.4	107 (70.9)	67.7	82.2		137 (90.7)	78.7	93.9
Healthy individuals	254	29 (11.4)			51 (20.08)				37 (14.6)		
Controls	43	11 (25.5)			23 (53.49)				7 (16.3)		
China panel											
NPC patients	145	122 (84.1)	91.7	81.6	125 (86.2)	93.3	83.9	183	174 (95.1)	95.6	89.3
Healthy individuals	113	11 (9.7)			9 (8.0)			83	8 (9.6)		

between both Chinese and Indonesian populations in the single-test EBNA1+VCA-p18 combination ELISA, which illustrates the diagnostic potential of this single-test option for NPC diagnosis and screening in both populations.

Immunoblot confirmation of discrepant samples. In order to further define the serological status of control samples with mostly weak OD_{450} values in IgA ELISA but above the CoV (37/252 healthy donors and 7/43 non-NPC patient controls) or below the CoV (for 14/151 NPC patients), the EBV IgG diversity pattern was determined by immunoblot analysis, recently shown by us to provide excellent discrimination of NPC and non-NPC patients from different geographical backgrounds (13). Figure 5A and B show IgG immunoblot patterns of 12 representative healthy controls and all 7 non-NPC patient controls, respectively. Thirty-four of 37 healthy carriers and 5 of 7 non-NPC tumor control patients with positive EBV IgA ELISA result showed a restricted IgG diversity pattern characteristic for healthy EBV carriers. These individuals had IgG reactivity only with EBNA1 (BKRF1; 72 kDa), VCA-p40 (BdRF1, 40 to 42 kDa), and VCA-p18 (BFRF3; 18 kDa), whereas one showed an additional weak reactivity to the ZEBRA protein (BZLF1; 36 and 38 kDa) (Fig. 5A, lane 3). Two of the non-NPC tumor patient controls showed increased IgG reactivity with the major EAd marker BMRF1 (47 to 54 kDa), as shown in Fig. 5B, lanes 5 and 7, suggestive of reactivating EBV infection. These data reveal that (weakly) increased EBV IgA ELISA reactivity in control EBV carrier populations is rarely associated with increased IgG diversity, confirming our previous studies.

On the other hand, 14 of 151 NPC samples (9.3%) showed IgA ELISA values below the CoV. Ten of these (71%) showed a complex IgG recognition pattern, previously established as the "NPC pattern" (collectively represented by lane 5 in Fig. 5C). Such an NPC pattern includes additional IgG reactivities to EBV EA proteins such as EA-p138 (BALF2; 138 kDa), thymidine kinase (BXLF1; 65 kDa), DNase (BGLF5; 55 and 58 kDa), and major-EA(D) (BMRF1; 47 to 54 kDa). Only 2/151 (1.3%) showed reactivity similar to most healthy donors and non-NPC controls (Fig. 5C, lanes 1 and 2). An additional two NPC patients showed restricted diversity but with increased intensity (Fig. 5C, lanes 3 and 4). Interestingly, immunoblotting revealed that three patients (Fig. 5C, lanes 1, 2, and 4) had no/low IgG response to EBNA1 and two patients (Fig. 5C, lanes 1 and 3) had no/low IgG responses to VCA-p18. These four sera also lacked IgA immunoblot reactivity to both markers (data not shown).

By including IgG immunoblotting as a confirmation assay for samples with EBV IgA values above the CoV, the sensitivity and specificity of EBV IgA ELISA for identification of true NPC cases increased from 85.4% to 98.6% and from 90.1% to 99.6%, respectively, and PPV/NPV values increased from 78.7/93.9% to 98/99.2%.

EBV IgA ELISA in acute EBV infection. In order to further define the clinical specificity of EBV peptide-based IgA

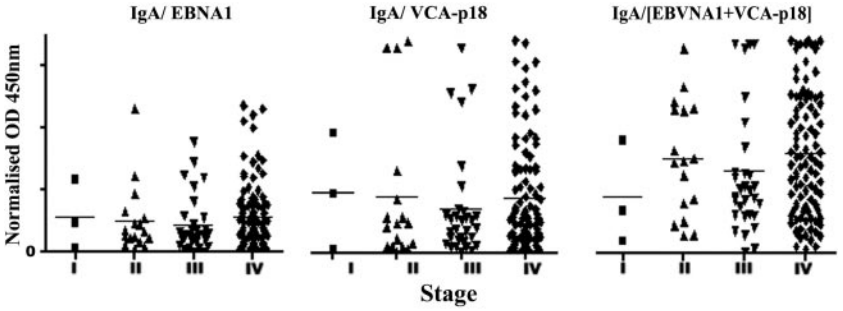

FIG. 4. Data analysis of IgA ELISA by using EBNA1 and VCA-p18 as single and combination antigens in NPC patients grouped by stage. IgA ELISA results for pretreatment sera of Indonesian NPC patients having stage I ($n = 3$), II ($n = 17$), III ($n = 31$), and IV ($n = 100$) disease at first presentation are shown. Although peak IgA EBV reactivities increase with stage, the mean OD_{450} values were not significantly different between different stages ($P > 0.05$).

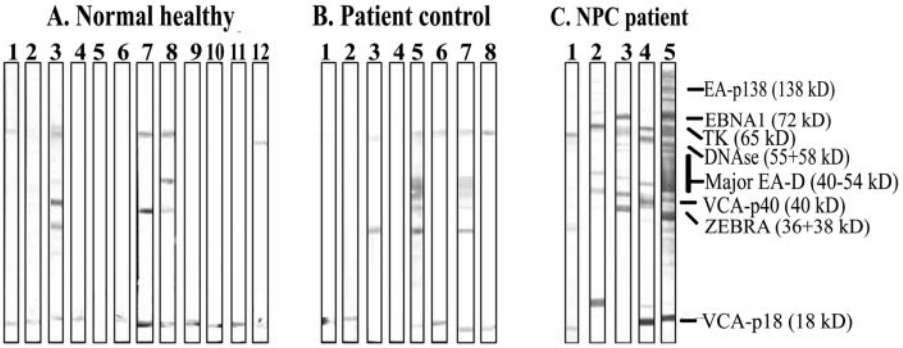

FIG. 5. IgG EBV immunoblot analysis detection for confirmation of false-positive and negative results in IgA EBNA1+VCA-p18 ELISA. Three of 37 healthy subjects and 2 of 7 patient controls with high OD_{450} showed slightly "abnormal patterns," while 2 of 14 NPC samples with low OD_{450} values showed "normal patterns" (for details see the text).

ELISA, we analyzed sera from patients with acute or chronic EBV infection, which may share some of the symptoms associating with early-stage NPC disease. EBV IgA ELISA using the combination of EBNA1+VCA-p18 peptides was therefore used on a panel of sera from patients with IM and C-EBV of European origin. Figure 6 shows that none of the sera from IM patients ($n = 39$) showed significant IgA reactivity to the combined EBNA1+VCA-p18 antigens, and only 3 of 10 (30%) C-EBV patients showed a positive IgA result. These results support the use of single-well IgA EBNA1+VCA-p18 ELISA for the specific diagnosis of NPC.

DISCUSSION

A novel one-step EBV IgA ELISA was developed by employing two synthetic peptides derived from EBNA1 and VCA-p18 antigens for the serodiagnosis of NPC in high-risk populations in Southeast Asia. This study extends a recent immunoblot study (13), which explored the molecular diversity of anti-EBV IgG and IgA responses in NPC patients from different geographical backgrounds. In that study we noticed distinct differences in the antigen fine specificity of IgG and IgA antibody responses, suggesting independent antigen triggering of B cells. In addition, significantly different antigen recognition profiles were revealed for both IgG and IgA antibody responses between NPC patients and regional controls, permitting diagnostic use (13, 23). Immunodominant proteins were defined, including VCA-p18 and EBNA1, and reactive epitopes were identified using PEPSCAN technology (31, 32, 43, 44). In this study we describe in detail the IgA responses against two synthetic multiepitope peptides previously proven of value for EBV-specific IgM- and IgG-based diagnosis of acute primary EBV infection (28, 32, 44).

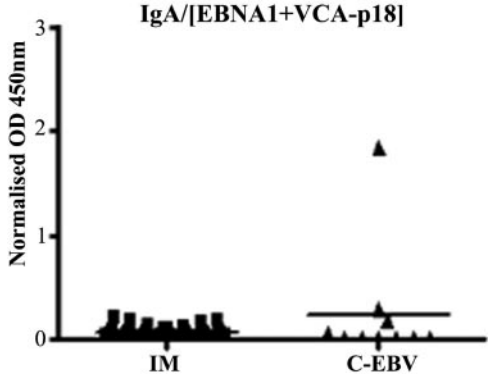

FIG. 6. IgA EBNA1+VCA-p18 ELISA of acute EBV infections. EBNA1+VCA-p18 combination peptide IgA ELISA was used for analyzing EBV IgA reactivity in sera from patients with acute IM ($n = 39$) or C-EBV ($n = 10$). Data show the absence of significant EBV IgA reactivity in IM patients and variable reactivity in about 30% of C-EBV patients in this assay.

Studies on NPC serodiagnosis have been described extensively in recent years. For example Hsu et al. (21) employed a recombinant EA and EBNA1 IgA ELISA for NPC diagnosis and screening in Taiwan and showed sensitivity, specificity, and accuracy of 98.1, 81.8, and 88.7%, respectively, for diagnosis of NPC patients and showed that the IgA ELISA values correlated with stage of disease. A more comprehensive ELISA study was done by Dardari et al. (9) with a panel of Moroccan NPC patients; this study employed multiple ELISA tests for detecting IgG and IgA response to VCA-p23-p18, EA-p54-p138, and EBNA1, in comparison to IFA IgG and IgA to VCA and EA. Their results showed that IgA EAd-p54-p138 ELISA had better diagnostic value for NPC detection (70%), compared with IgA VCA-p18-p23 and IgA EBNA1 ELISA, which particularly had limited diagnostic value in young patients. Furthermore IgA EAd-p54-p138 ELISA could detect 64% of NPC cases negative by classic IFA. The combination of IgG ZEBRA IFA with IgA EAd-p54-p138 ELISA improved the sensitivity of detection of NPC to 95% in the overall NPC population. Similarly, Chen et al. (4) generated an EBNA1 recombinant derived from an NPC biopsy and developed an IgA EBNA1 ELISA with a 78.7% positivity rate for NPC samples, which could be raised to 92.5% when combined with IgA VCA using IFA. More recently, Karray et al. (23), using identical EBV synthetic peptides as in this study, demonstrated excellent correlation between individual EBV peptide-based ELISAs and classical IFA-based serology and showed that IgA VCA p18 peptide ELISA raised the detection rate from 78.6% to 89.8% in young Tunisian NPC patients compared to IFA. These studies, however, all showed that a single serological test cannot achieve the ideal objective of identifying all NPC patients, and it was advised to combine different tests for diagnosis of NPC. Combinations of different technologies, how-

ever, add to cost and may be difficult to realize in developing countries. A single-assay format is preferred. Peptides may substitute for natural or recombinant proteins as a stable, reproducible, and cheap source of antigen in ELISA. VCA-p18, the small capsid protein of 18 kDa encoded by the BFRF3 gene, is highly immunogenic in humans, and its use in ELISA is well described (3, 9, 30, 35, 44). The EBNA1 synthetic peptide used in this study derived from a unique region of EBNA1 (combining amino acids 382 to 410 and 413 to 452) not including glycine-alanine repeat sequences, which may produce nonspecific reactivity (27, 32).

Compared to the studies mentioned above, we here show that a synthetic-peptide-based EBV IgA ELISA, combining VCA-p18 and EBNA1 epitopes into a single ELISA well, can achieve sensitivity and specificity values above 85% in two independent NPC populations from Indonesia and southern China. Importantly, this assay not only allows discrimination between NPC patients and healthy EBV carriers but also between NPC and non-NPC tumor patients in regions with high NPC prevalence. Importantly, we show that the EBV IgA EBNA1+VCA-p18 combination peptide ELISA permits discrimination of NPC patients from patients with acute or chronic active EBV infection, which may present with similar nonspecific symptoms. This is relevant for diagnostic screening in populations with symptoms in head and neck suspicious of NPC. Therefore this well-defined assay may be suitable for large-scale diagnostic screening in high-risk regions. However, we failed to find a direct correlation between EBV IgA reactivity and TNM stage, as was found in another study (21). This might be related to the relatively high sensitivity of the VCA-p18+EBNA1 peptide combination ELISA for detecting early-stage NPC, but this remains to be proven in more-extended studies, because early-stage samples were rare in our population.

Our data reveal that removal of IgG by using Gullsorb is not required for optimal EBV IgA detection by ELISA, indicating that IgG and IgA may react with different epitopes or that IgA avidity is sufficient to compete with IgG. This is in line with our previous data showing that IgG and IgA responses have distinct EBV antigen specificities and may be triggered differently (13). We show by comparing the IgG and IgA ELISA results for EBNA1 and VCA-p18 peptide antigens either alone or in combination that the IgG levels in all three populations were higher than IgA, being highest in NPC patients ($P < 0.05$), with 100% of tested individuals having a positive result in the EBNA1+VCA-p18 combination test. The 100% EBV IgG-positive rate is not surprising, since in Asia most individuals acquire EBV during early childhood (20, 48). However, the considerable overlap in OD_{450} values in EBV IgG combination peptide ELISA between healthy donors and NPC patients precludes its diagnostic use in the NPC setting.

On the other hand, mean EBV IgA antibody levels in the NPC panel were significantly higher ($P < 0.0001$) that those of healthy blood donors and control patients, either in single- or double-antigen ELISA (Fig. 3). Sensitivity of combined EBNA1+VCA-p18 peptide ELISA (85.3%) was lower than EBNA1-only ELISA (88.5%), but it showed higher specificity (90.1%) compared to either EBNA1 or VCA-p18 as a single antigen. The relatively high reactivity of IgA VCA-p18 in Indonesian non-NPC patients may be explained by (subclinical) EBV reactivation and local EBV replication in the nasopharynx (51) due to poor health conditions, similar to recent observations by us for human immunodeficiency virus carriers (39). EBNA1 IgA responses appear to be more specific for NPC and may be related to release of EBNA1-DNA complexes from dying NPC tumor cells (40). However, not all NPC patients have a detectable EBNA1 IgA response, and combination with VCA-p18 in an IgA ELISA clearly improves diagnostic use.

In order to increase the specificity and sensitivity of serological diagnosis of NPC, we employed a "second-line" IgG immunoblot test as a confirmatory assay. In NPC patients, IgG antibodies typically react with a broad range of EBV lytic proteins (13). By combining EBV IgA ELISA with IgG immunoblot analysis of samples showing an initial positive ELISA result, specificity/sensitivity and PPV/NPV for NPC detection increased to >95%. Using the current EBNA1+VCA-p18 peptide mixture some 9% of NPC patients would be missed in an IgA ELISA-based screening program, and ideally these patients should be identified. Immunoblot analysis revealed 4/14 (29%) of these nonresponders indeed had low-to-undetectable EBNA1 of VCA-p18 IgG and IgA responses, confirming the ELISA results. However, additional IgA-reactive bands were identified for most of these sera, which are mainly characterized as EA complex proteins (Fig. 5C). Further work is in progress to define additional IgA-reactive proteins and epitopes and to further increase the overall sensitivity of the synthetic-peptide ELISA approach for primary NPC screening in high-risk populations.

Overall, the combined IgA EBNA1+VCA-p18 ELISA showed promise as a tool for primary diagnosis of NPC in Indonesian and Chinese populations compared to a single-antigen IgA ELISA. It provides an important step to development of a well-standardized and affordable test for use in high-risk populations. The lack of significant IgA responses to EBNA1 and VCA-p18 combination peptides in acute and chronic EBV infection further supports the potential use of EBV IgA combination peptide ELISA as an NPC-specific screening tool. The inclusion of a confirmation assay is advocated for individuals with a positive result in the initial IgA ELISA screening. The immunoblot technique or EBV DNA load analysis may well fulfill this purpose (2, 13, 23, 24, 26). Importantly, it was recently shown that EBV DNA load measurement may provide an independent marker for NPC, not related to serology (24, 40). A combined positive result in independent tests may be used to select individuals for detailed clinical examination including endoscopy and biopsy. Finally, it should be stressed that previous large-scale seroepidemiological studies in China and Taiwan revealed that apparently healthy individuals with elevated EBV IgA responses have significantly increased risk for NPC development in subsequent years (6, 10). Such individuals may also be included among the healthy donors with positive EBV IgA ELISA result identified here. However, this awaits clinical confirmation in more detailed follow-up studies.

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